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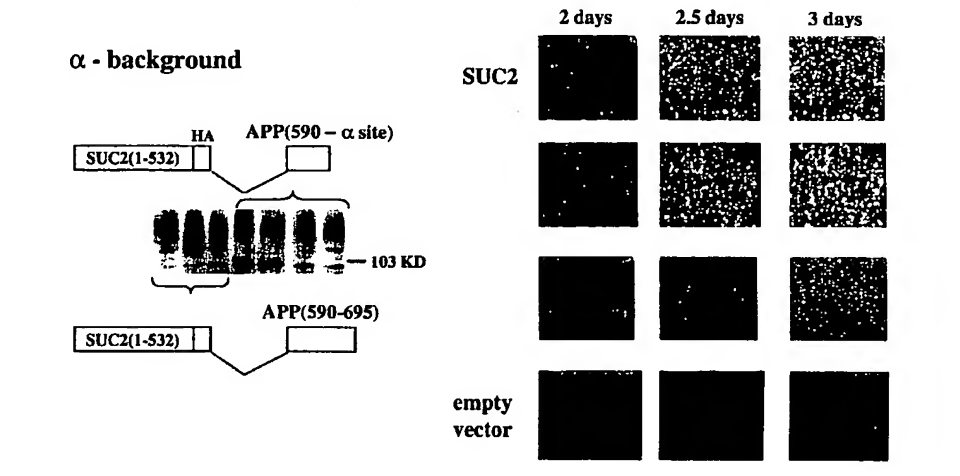
(54) Title: METHOD FOR IDENTIFY POLYPEPTIDES WITH PROTEASE ACTIVITY



(57) Abstract: The present invention describes methods for the identification of proteins with protease activity and methods for the analysis of protein-protein interactions of membrane proteins.

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METHOD FOR IDENTIFY POLYPEPTIDES WITH PROTEASE ACTIVITY

Technical Field

The present invention relates to methods for the identification of proteins with secretase activity and methods for the analysis of interactions between membrane proteins.

Background Art

Protein secretion is central to the proper development and function of eucaryotic organisms. Moreover, several pathophysiological processes such as neurodegeneration, oncogenesis, apoptosis and inflammation are associated with the malfunction or aberrant regulation of protein secretion. It has become clear that there is no single biosynthetic mechanism common to all secretory proteins. Secretion of proteins can occur through either the regulated or constitutive pathways and, in some cell types, this secretion can be polarized to distinct cellular domains. An increasing number of proteins are now recognized as being derived from integral membrane proteins of type I and type II topology and, in this case, the secretory event involves their selective post-translational hydrolysis from the cell surface. This secretion is catalyzed by proteases known as secretases. The cleavage of membrane proteins generally occurs near the extracellular face of the membrane, although in some cases it has been shown also to occur within the transmembrane domain. Proteins secreted in this fashion include membrane receptors and receptor ligands, ectoenzymes, cell adhesion molecules and others. Examples of protein secretion through the action of secretases include the vasoregulatory enzyme ACE (angiotensin converting enzyme), the tumor necrosis factor (TNF) ligand and receptor superfamilies, the transforming growth factor-α, certain cytokine receptors, the Alzheimer's amyloid precursor protein (APP) and

others (Hooper, N.M., Karran, E.H., and Turner, A.J. (1997) Biochem. J. 321, 265-279). Most secretases have so far eluded identification and cloning. Evidently, the cloning of the genes encoding these enzymes would dramatically facilitate their characterization and provide the unique condition to design specific inhibitors or stimulators that target discrete secretases.

In the past, different methods have been used to clone genes encoding secretases. The most widely used method has been purification of the protein and subsequent isolation of the corresponding cDNA sequence (Black, R.A et al., (1997) Nature 385, 729-733; Moss M.L. et al., (1997) Nature 385, 733-736; Howard L. et al., (1996) Biochem. J. 317, 45-50)). An alternative method has been used by Yan et al., who have cloned a APP β-secretase by exploiting pharmacological data, which allowed classification of this secretase as an aspartyl protease, to scan the almost completely sequenced genome of the nematode worm *C. elegans* for candidate protease genes, and to subsequently use such candidates to identify human homologues (Yan R. et al., (1999) Nature 402, 533-537). A third method has been applied by Vassar et al., who have cloned the same β-secretase gene by screening pools of human cDNAs which were expressed in a mammalian cell line and analyzed for increased release of the APP β proteolytic product (Vassar R. et al., (1999) Science 286, 735-741).

It is already known from Hawkins et. al., (Hawkins C.J., Wang S.L., and Hay B.A., (1999) Proc.Natl.Acad.Sci.USA 96, 2885-2890) to use yeast for screening for cytoplasmic proteases of the group of caspases and their regulators. In the described method a fusion protein is created in which a transcription factor is linked to the intracellular domain of a transmembrane protein by caspase cleavage sites. The transcription factor is part of a reporter system thus that in the

presence of a caspase the transcription factor is released and induces transcription of a reporter gene and thus allows identification of positive cells. The method allows only the isolation of members of the caspase group.

In the past various methods were developed to identify protein-protein interactions in vivo. The two hybrid system is a very powerful tool for the in vivo analysis of interactions between soluble proteins (Bartel P.L., and Fields S., (1995) Methods Enzymol., 254, 241-263). The split-ubiquitin system is and alternative method for the analysis of interactions between soluble proteins (Johnsson N., and Varshavsky A., (1994) Proc.Natl.Acad.Sci.USA, 91, 10340-10344). Stagljar et al., (Stagljar I, Korostensky C., Johnsson N., and Te Heesen S., (1998) Proc.Natl.Acad.Sci.USA, 95, 5187-5192) describe a genetic system based on split-ubiquitin for the analysis of interactions between membrane proteins in vivo. This method provides a potentially useful tool for screening of interaction between membrane proteins.

The importance of secretases in many biological processes makes them attractive novel targets for the development of therapeutic drugs and this raises the need for reliable screening systems for the cloning of new members of the secretase family which are more sensitive and efficient than the known traditional methods.

Disclosure of the Invention

Hence, it is a general object of the present invention to provide a method for the identification of a secretase wherein suitable host cells are transformed to express under suitable conditions a target membrane protein as a fusion protein with a secreted protein and said suitable host cells transformed to express said fusion protein are further transformed with a library encoding candidate secretases and said host cells

transformed with said fusion protein and said library are cultivated under conditions allowing expression of both said fusion protein and said library and said conditions allowing cell survival only in presence of said secreted protein that has been released from said fusion protein by a secretase encoded by said library.

The present invention furthermore provides a secretase that can be obtained by a method of the invention. The term secretase as used herein should be understood to include all types of proteins with protease activity.

Another object of the present invention relates to a method for the identification of a membrane protein that is a substrate of a known secretase wherein suitable host cells are transformed to express under suitable conditions said secretase and said host cells transformed to express said secretase are further transformed with a library encoding fusion proteins with a secreted protein and a candidate substrate and said host cells are cultivated under conditions allowing expression of both the secretase and the library encoded fusion protein and allowing cell survival only in the presence of said secreted protein that has been released from said fusion protein by said secretase due to the interaction of said secretase with said library encoded substrate.

Another object of the present invention concerns a method for screening for a protein interacting with a target membrane protein wherein suitable host cells are transformed to express under suitable conditions said membrane protein as a fusion protein with a secreted protein and the two moieties of said fusion protein are linked by a recognition sequence that is cleaved by a defined secretase. Said host cells transformed to express said fusion protein are further transformed with a library encoding fusion proteins with said defined secretase and candidate interaction partners

of said known membrane protein and cultivated under conditions allowing expression of both the fusion protein and the library and allowing cell survival only in the presence of an interaction of the known membrane protein with the library encoded interaction partner protein. Due to the interaction of said known membrane protein and the library encoded partner protein the secretase of said library fusion protein is brought into vicinity of said secretase recognition sequence linking the target membrane protein and the secreted protein and this vicinity allows the release of the secreted protein from said fusion protein and thus cell survival.

Appropriate culturing conditions of the cells must be used such that said fusion protein is not efficiently cleaved by the secretase in the absence of a protein-protein interaction between said known membrane protein and said library encoded partner protein.

In a preferred embodiment said secretase is modified, preferably said modification is such that the membrane-anchoring domain of said secretase is deleted.

This method allows the identification of interactions between membrane bound proteins or between ER/Golgi luminal proteins.

The invention furthermore provides an interacting protein that can be obtained by a method of the invention.

Another object of the present invention is a method for screening for a secretase wherein suitable host cells are transformed to express under suitable conditions a target membrane protein as a fusion protein comprising the known membrane protein, a secreted protein and a transcriptional activator which is part of a reporter system that is stably integrated into the genome of said host cells. Said host cells are further transformed with a library encoding candidate secretases and cultured under conditions allowing expression of both said fusion protein and said library and allowing cell

survival and selection of positive cells only in the presence of a library encoded secretase that cleaves said fusion protein and thus releases said secreted protein and the transcription activator from said fusion protein thus allowing cell survival and activation of a reporter gene and thus selection of positive cells.

The presence of a secreted protein and a transcriptional activator in a fusion protein with a target transmembrane protein allows the rapid identification of secretases cleaving a transmembrane protein in its transmembrane region. In such a case both the secreted protein is released from the fusion protein and can be secreted thus allowing cell survival and the transcriptional activator is released and can enter the cell nucleus and induce transcription from the reporter gene construct and thus allowing selection of positive cells.

Protein-protein interactions can, preferably, be monitored by a transcriptional readout, preferably by the expression of the HIS3 gene whose expression is under control of said transcriptional activator. Many transcriptonal activators useful for the described methods are well known to the skilled person and are for example LexA-VP16.

The invention provides furthermore a secretase that can be obtained by a method of the invention.

Secretases are generally expressed as pro-enzymes which are dependent on processing to be activated. The need of an external processing activity exists when the prosecretase is not capable of autoprocessing. Therefore the present invention provides a method for the identification of a protein that is able to process a specific secretase wherein suitable host cells are manipulated to express under suitable conditions a target membrane protein as a fusion protein with a secreted molecule and a unprocessed specific

secretase. Said cells are further transformed with a library encoding candidate processing proteins of said secretase and cultivated under conditions allowing expression of said fusion protein, said unprocessed specific secretase and said library and said conditions furthermore allowing cell survival only in the presence of said secreted protein that has been released from said fusion protein by said secretase that has been processed by the library encoded processing protein.

In a preferred embodiment of all the above mentioned objects of this invention the host cell is an eucaryotic cell, in particular a yeast cell. The target membrane protein preferably is a transmembrane protein, preferably a type I or a type II transmembrane protein. The term membrane protein as used herein comprises full length proteins as well as fragments thereof. Depending on the particular topology of the membrane protein the secreted protein moiety is fused to the N-terminus or the C-terminus of said protein, such that said moiety faces the ER lumen. General molecular biological and biochemical methods well known to the person skilled in the art are applied to determine the most suitable fusion protein of the target membrane protein and the secreted protein to result in a functional secreted protein after secretase cleavage (Maniatis et al., Molecular Cloning: A Laboratory Manual, New York:Cold Spring Harbor Laboratory, 1989). There are many strategies well known in the technical field to construct DNA libraries appropriate for the purposes of the present invention and to clone them in suitable vectors for expression in host cells (Maniatis et al., Molecular Cloning: A Laboratory Manual, New York:Cold Spring Harbor Laboratory, 1989).

The introduction of the expression constructs of this invention into said suitable host cells can be performed by cotransformation or more preferably by sequential transformation, wherein in a much preferred embodiment of this invention said host cells are first

transformed with the expression construct encoding said target protein or a fusion protein of said target protein and in a second transformation step said cells transformed to express said target protein or a fusion protein of said target protein are transformed with the second expression construct. The second construct can be a library encoding fusion proteins or a library encoding single proteins.

In a preferred embodiment of the present invention the secreted protein is an protein with invertase activity or functional fragments of a protein with invertase activity, preferably a yeast invertase or functional fragments of a yeast invertase. Yet it is obvious for the man skilled in the art that other secreted proteins e.g the yeast pheromone α-peptide, can be used in the scope of the present invention as a reporter system.

Other suitable host cells for all the objects of this invention are tissue culture cell lines whose growth is dependent on a protein growth factor. These cells can be manipulated such that they express in one embodiment of this invention the growth factor fused to a target membrane protein. Since the modified growth factor can not be secreted, these cells are dependent on a exogenous growth factor. Said host cells are then further transfected with a library and cultivated under conditions allowing the expression of both said fusion protein of the target membrane protein with said growth factor and the library and said conditions furthermore allowing growth of said host cells only in the presence of said growth factor that has been released from said fusion protein by a library encoded secretase. A suitable host cell line is for example PC12 whose growth is depending on the presence of NGF (nerve growth factor).

Brief Description of the Drawings

The invention will be better understood and objects other than those set forth above will become apparent when consideration is given to the following detailed description thereof. Such description makes reference to the annexed drawings, showing the following:

Fig 1 A: Deletion strategy of the YAP3 gene of S.cerevisiae.

Fig 1 B: Confirmation of homologous recombination at the YAP3 locus by PCR genotyping.

Fig 1 C: Deletion strategy of the MKC7 gene of S.cerevisiae.

Fig 1 D: Confirmation of homologous recombination at the MKC7 locus by PCR genotyping.

Fig 2 A: Deletion strategy of the SUC2 gene in S. cerevisiae.

Fig 2 B: Confirmation of homologous recombination at the SUC2 locus by PCR genotyping.

Fig 3: Genetic test for suc2 disruption

Fig 4 A: Western blot analysis of SUC2 fusion with N-terminal truncated APP.

Fig 4 B: Colony formation of ULY 2 cells expressing SUC2-APP fusion proteins on sucrose plates.

Fig 5: SUC2-APP(590-695) is activated in α+ background.

Fig 6: ER retrieval signal in α+ and α- background.

Modes for Carrying Out the Invention

The ability of yeast such as *Saccharomyces cerevisiae* to utilize sucrose as a carbon source depends on the secretion of the enzyme invertase, which cleaves sucrose to yield glucose and fructose (Carlson, M. et al. (1983) Mol. Cell. Biol. 3, 439-447). Indeed, deletion of the entire SUC2 gene, which encodes the invertase protein, or deletion of the signal peptide, which prevents secretion of the invertase, cripples the ability of yeast cells to grow on sucrose medium (Perlman, D.,

and Halvorson, H.O. (1981) Cell 25, 525-536; Carlson, M., and Botstein, D. (1982) Cell 28, 145-154; Kaiser, C.A., and Botstein, D. (1986) Mol. Cell. Biol. 6, 2382-2391). Thus, the manipulation of invertase expression and secretion provides a convenient genetic selection for engineered yeast cells, as it has been shown by two research groups who have utilized this growth selection system to isolate human cDNAs encoding secreted proteins (Klein, R.D. et al. (1996) Proc. Natl. Acad. Sci. USA 93, 7108-7113; Jacobs, K.A. et al. (1997) Gene 198, 289-296; patents: US005536637A, US00571211A, WO 97/40146). In these works, the authors have used yeast strains deleted for the endogenous SUC2 gene to express human cDNAs fused to a modified SUC2 gene lacking its leader sequence, which encodes the secretion signal sequence. Heterologous secreted proteins appropriately fused to the N-terminus of the modified, non-secreted invertase could be identified through positive selection because they provided the necessary signals to restore invertase secretion, thus restoring cell growth on sucrose medium.

Expression and localization of the invertase protein have also been monitored by alternative techniques such as colorimetric (Goldstein, A., and Lampen, O.J. (1975) Methods Enzymol. 42, 504-511) and immunodetection assays. For example, these techniques have been used to identify protein-sorting sequences that mediate localization to yeast mitochondria (Emr, S.D. et al. (1986) J. Cell. Biol. 102, 523-533), vacuoles (Klionsky, D.J. et al. (1988) Mol. Cell. Biol. 8, 2105-2116; Tague, B.W. et al. (1990) Plant Cell 2, 533-546), or endoplasmic reticulum (Gaynor, E.C. et al. (1994) J. Cell. Biol. 127, 653-665; Boehm, J. et al. (1994) EMBO J. 13, 3696-3710), to determine the effects of mutations within signal sequences (Ngsee, J.K. et al. (1989) Mol. Cell. Biol. 9, 3400-3410), and to monitor the amounts of human proteins expressed from yeast (Hitzeman, R.A. et al. (1990) Methods Enzymol. 185, 421-440).

In one embodiment of the present invention, the identification of a secretase activity expressed in yeast is based on its ability to cleave a specific target membrane protein fused to the invertase enzyme. Because of its fusion with the membrane-bound protein, this invertase is not secreted; consequently, these yeast cells, which lack the endogenous invertase, cannot grow on sucrose medium. However, in the presence of a secretase activity that specifically recognizes and cleaves the membrane-bound protein, the invertase enzyme is liberated from its anchor and it is secreted to the periplasm where it can hydrolyze sucrose, thus allowing cell growth on sucrose medium.

For the experiments described here, chimerical proteins were used bearing the invertase enzyme fused to different portions of the membrane-bound Amyloid-β Precursor Protein (APP). In human cells, APP, a type I transmembrane protein, can be processed by three types of proteases denoted α, β- and γ-secretases. Cleavage by the latter two generates the 40 and 42 amino acids Aβ peptides involved in Alzheimer's disease, while the α-secretase cleaves APP near the middle of the Aβ sequence. Cleavage of APP by α and β-secretases occurs at the luminal/extracellular face of the membrane, whereas cleavage by γ-secretase has been shown to occur within the transmembrane domain of APP. APP expressed in the yeast *Saccharomyces cerevisiae* is efficiently processed by endogenous proteases which cleave the protein almost exclusively at the α-site. Two research groups have independently shown that the glycosyl-phosphatidylinositol-linked aspartyl proteases Yap3p and Mkc7p are primarily responsible for α-secretase-type cleavage of APP, which results in release and secretion of soluble APP into the periplasm (Zhang, W. et al. (1997) Biochim. Biophys. Acta 1359, 110-122; Komano, H. et al. (1998) J. Biol. Chem. 273,31648-31651). Deletion of YAP3 and MKC7 in a vacuolar protease-deficient strain

abolished α-secretase cleavage, which could be restored by reintroducing MKC7 or YAP3 on single copy plasmids (Komano, H. et al. (1998) J. Biol. Chem. 273,31648-31651). Here we present results showing that a yeast strain deleted for the endogenous SUC2 gene and expressing the invertase enzyme fused to the membrane-bound APP can efficiently grow on sucrose only when the Yap3p and Mkc7p secretases that cleave APP at the α-site are co-expressed with the fusion protein.

The experiments utilize two *Saccharomyces cerevisiae* strains, both of which have been deleted for the SUC2 gene, and one of them has been additionally deleted for the YAP3 and MKC7 genes. Fig. 1 shows the strategy that was used to delete the YAP3 and MKC7 genes. For the YAP3 knock-out, a kanamycin resistance cassette specifically constructed for yeast expression (Steiner, S., and Philippsen, P. (1994) Mol. Gen. Genet. 242, 263-271) was amplified by PCR using primers that possess at their 5′ termini sequences homologous to YAP3 regions (Fig. 1A). The yeast strain JPY9 (Barberis, A. et al. (1995) Cell 81, 359-368) was transformed with this PCR product and plated on a selective agar medium containing the kanamycin analog G418. Only those cells that have steadily integrated the kanamycin resistance cassette could grow and form colonies on these selective plates. In order to check whether integration had occurred in a homologous manner, namely at the YAP3 locus, we performed genotyping by PCR using two sets of primers (Fig. 1B). The yap3 derivative of JPY9 was named STY1. To knock out the MKC7 gene in STY1, a functional HIS3 gene-cassette was amplified by PCR using primers that bear at their 5′ termini sequences homologous to the MKC7 gene (Fig. 1C). STY1, which lacks the endogenous HIS3 gene, was transformed with this PCR product and plated on a selective agar medium lacking histidine. Only those cells that have steadily integrated the HIS3 gene-cassette could grow and form colonies on these selective plates.

In order to check whether integration had occurred in a homologous manner, namely at the MKC7 locus, we performed genotyping by PCR using three sets of primers (Fig. 1D). The yap3, mkc7 double knock-out strain was named STY2. Fig. 2 shows the strategy we used to knock out the SUC2 gene in both STY1 and STY2 strains. A functional URA3 gene-cassette was amplified by PCR using primers that bear at their 5' termini sequences homologous to the SUC2 gene (Fig. 2A). The yeast strains JPY9 and STY2, which lack a functional URA3 gene, were transformed with this PCR product and plated on a selective agar medium lacking uracil. Only those cells that have steadily integrated the URA3 gene-cassette could grow and form colonies on these selective plates. In order to check whether integration had occurred in a homologous manner, namely at the SUC2 locus, we performed genotyping by PCR using two sets of primers (Fig. 2B). The suc2 derivative of JPY9 was named ULY1, while the suc2 derivative of STY2 was named ULY2.

The phenotype of the suc2 strains was tested as follows. The SUC2 gene was cloned on a single copy yeast expression vector and introduced into the strains ULY1 and ULY2. Growth of the transformed cells on sucrose plates was monitored at different time points and compared to growth of the same strains that were transformed with a vector that did not bear the SUC2 gene. Fig. 3 shows that, while growth on sucrose plates of the strains lacking SUC2 was dramatically impaired, expression of the vector-borne SUC2 gene complemented the suc2 phenotype. The growth rate of the complemented strains was indistinguishable from that of wild type cells (data not shown). Moreover, no significant growth difference was observed between ULY1 and ULY2 in this experiment.

The invertase-APP fusion protein tested in the experiments described here is composed of full-length invertase (amino acid residues 1-532) sequentially

followed by a 9 amino acid HA-tag and by a C-terminal portion of APP (amino acid residues 590-695), which contains, in addition to the transmembrane domain (aa 622-646), the cleavage sites for all three mammalian secretase (β between aa 596 and 597, α between aa 612 and 613, and γ between aa 636 and 637 or between aa 638 and 639). It has been shown that an APP deletion mutant containing amino acid residues 590 to 695 is proteolytically processed in a proper manner in mammalian cells (Citron, M. et al. (1995) Neuron 14, 661-670). In order to control and predict the activity of the invertase-HA-APP fusion which is expected to be secreted upon cleavage by the α-secretases in yeast, we constructed an invertase-HA-APP fusion protein bearing an APP sequence ending at the α site (aa 590-612). Expression of both fusion proteins in the strain ULY2, which lacks α-secretase activities, was confirmed by Western blot analysis using anti-HA antibodies (Fig. 4A). Fig. 4B shows the effect that these fusion proteins had on the rate of ULY2 cell growth and colony formation on sucrose plates. As a positive control, the SUC2 expression plasmid described above was used; an empty vector, i.e. not expressing any SUC2 gene, was used as a negative control. Growth on sucrose plates of ULY2 cells expressing either the wild type SUC2 gene or the α-truncated invertase-HA-APP (590-612) fusion protein was indistinguishable (Fig. 4B, top two rows), indicating that fusion of this portion of APP to the C-terminus of the invertase did not influence invertase function. On the other hand, expression of the invertase-HA-APP (590-695) fusion protein, which bears the transmembrane domain, visibly reduced growth of the transformed ULY2 cells, although not to the extent observed with cells transformed with the empty vector (Fig. 4B, bottom two rows).

In order to test the hypothesis that co-expression of the yeast α-secretases Yap3p and Mkc7p with

the invertase-HA-APP(590-695) fusion protein would cause cleavage of the APP sequence at the α site, thus allowing efficient secretion of the invertase, we compared growth on sucrose plates of ULY1 (containing the α-secretases; α+) with ULY2 (lacking the α-secretases; α-) after transformation with the vector expressing the invertase-HA-APP(590-695) fusion protein. Fig. 5 shows that co-expression of active α-secretases with this invertase fusion protein in yeast cells lacking endogenous invertase restored their ability to grow on sucrose plates to an extent very similar to the positive control, in which cells were transformed with a vector expressing wild type invertase. In contrast, yeast cells that do not co-express the α-secretases with the invertase fusion protein have a reduced growth rate on sucrose plates, although, as previously shown, not to the extent observed with cells transformed with the empty vector.

One explanation for the observed residual activity of the invertase-HA-APP (590-695) fusion protein expressed in cells lacking Yap3 and Mkc7 α-secretases is that this membrane-bound fusion protein travels through the endoplasmic reticulum (ER) and Golgi to the plasma membrane, where the invertase moiety exposed to the periplasm could hydrolyse sucrose, thus allowing growth of the cells. To test this possibility, a ER retrieval signal (DEKKMP) was added to the C-terminus of the invertase-HA-APP (590-695) fusion protein. This signal has been shown to direct retrieval of transmembrane proteins from Golgi to the ER (Gaynor, E.C. et al. (1994) J. Cell. Biol. 127, 653-665). Fig. 6 shows that expression of this novel fusion protein in cells lacking the α-secretases did not confer them any ability to grow on sucrose plates above that observed upon transformation of an empty plasmid (negative control). However, co-expression of this fusion protein with the α-secretases restored the ability of the transformed cells to grow on

sucrose plates almost to the extend achieved by expressing wild type invertase.

While there are shown and described presently preferred embodiments of the invention, it is to be distinctly understood that the invention is not limited thereto but may be otherwise variously embodied and practiced within the scope of the following claims.

Claims

1. A method for the identification of one of a pair or a group of interacting proteins comprising

transforming suitable host cells such that under selective conditions they only survive in the presence of desired protein-protein interaction due to the release of at least one protein that is essential for cell survival under selective conditions, whereby at least one of said proteins essential for cell survival is a secreted protein that in the presence of desired interaction is released by cleavage of a fusion protein comprising said secreted protein by means of a suitable secretase.

2. The method of claim 1 wherein said proteins are a secretase and a protein comprising a cleavage site of said secretase, wherein

suitable host cells are transformed to express under suitable conditions both of said proteins,

whereby one of said proteins is a candidate protein of a library to be screened, and whereby

one of said proteins is present as a fusion protein with a secreted protein,

said host cells transformed with said proteins are cultivated under conditions allowing expression of both proteins, and

said conditions furthermore allow cell survival only in the presence of said secreted protein that has been released from said fusion protein due to secretase activity exerted on the substrate of said secretase.

3. The method of claim 1 or 2 wherein said interacting protein to be identified is a secretase and wherein

suitable host cells are transformed to express under suitable conditions a target membrane protein as a fusion protein with a secreted protein

said suitable host cells transformed to express said fusion protein are further transformed with a library encoding putative secretases

said host cells transformed with said fusion protein and said library are cultivated under conditions allowing expression of both said fusion protein and said library

said conditions furthermore allowing cell survival only in the presence of said secreted protein that has been released from said fusion protein by a secretase encoded by said library.

4. The method of claim 3, wherein the host cell is an eucaryotic cell.

5. The method of claim 3 or 4, wherein the host cell is a yeast cell.

6. The method of one of claims 3-5, wherein the target protein is a transmembrane protein.

7. The method of one of claims 3-6, wherein the target protein is a type I transmembrane protein.

8. The method of one of claims 3-6, wherein the target protein is a type II transmembrane protein.

9. The method of one of claims 3-8, wherein the secreted protein is a protein with invertase activity or functional fragments of a protein with invertase activity.

10. The method of one of claims 3-9, wherein the secreted protein is a yeast invertase or functional fragments of a yeast invertase.

11. A secretase obtainable by the method of one of claims 3-10.

12. A protein obtainable by the method of one of claims 3-10 as diagnostic, preventive or therapeutic agent.

13. The method of claim 1 or 2 wherein said interacting protein to be identified is a substrate of a known secretase and wherein

suitable host cells are transformed to express under suitable conditions said secretase

said suitable host cells transformed to express said secretase are further transformed with a library encoding fusion proteins of a secreted protein with candidate substrates of said secretase

said host cells transformed with said secretase and said library are cultivated under conditions allowing expression of both said secretase and said library

said conditions furthermore allowing cell survival only in the presence of said secreted protein released from said libray encoded fusion protein by said secretase.

14. The method of claim 13, wherein the host cell is an eucaryotic cell.

15. The method of claim 13 or 14, wherein the host cell is a yeast cell.

16. The method of one of claims 13-15, wherein the secreted protein is a protein with invertase activity or functional fragments of a protein with invertase activity.

17. The method of one of claims 13-16, wherein the secreted protein is a yeast invertase or functional fragments of a yeast invertase.

18. A protein obtainable by the method of one of claims 13-17.

19. The protein of claim 18 as diagnostic, preventive or therapeutic agent.

20. The method of claim 1 wherein said interacting protein to be identified is a protein interacting with a known target membrane protein and wherein

suitable host cells are transformed to express under suitable conditions said target membrane protein as a fusion protein with a secreted protein whereby the two moieties of said fusion protein are

linked by a recognition site forming a cleavage site that is cleaved by a defined secretase

said host cells transformed to express said fusion protein are further transformed with a library encoding fusion proteins of said defined secretase with candidate interaction partner proteins of said target protein

said host cells transformed to express said fusion protein and said library are cultivated under conditions allowing expression of both said target membrane fusion protein and said library

said conditions furthermore allowing cell survival only in the presence of said secreted protein released from said fusion protein by said defined secretase due to an interaction of the known target membrane protein with the library encoded interaction partner protein.

21. The method of claim 20, wherein the host cell is a eucaryotic cell.

22. The method of claim 20 or 21, wherein the host cell is a yeast cell.

23. The method of one of claims 20-22, wherein the target protein is a transmembrane protein.

24. The method of one of claims 20-23, wherein the target protein is a type I transmembrane protein.

25. The method of one of claims 20-23, wherein the target protein is a type II transmembrane protein.

26. The method of one of claims 20-25, wherein the secreted protein is a protein with invertase activity or functional fragments of a protein with invertase activity.

27. The method of one of claims 20-26, wherein the secreted protein is a yeast invertase or functional fragments of a yeast invertase.

28. A protein obtainable by the method of one of claims 20-27.

29. The protein of claim 28 as diagnostic, preventive or therapeutic agent.

30. The method of claim 1 or 2 wherein said interacting protein to be identified is a secretase and wherein

suitable host cells are transformed to express under suitable conditions a membrane protein as a fusion protein that comprises said membrane protein, a secreted protein and a transcriptional activator which is part of a reporter system that is stably integrated into the genome of said host cells

said host cells transformed to express said fusion protein are further transformed with a library encoding candidate secretases

said host cells transformed to express said fusion protein and said library are cultivated under conditions allowing expression of both said fusion protein and said library

said conditions furthermore allowing cell survival and selection of positive cells only in the presence of a library encoded secretase that cleaves said fusion protein and thus releases the secreted protein and the transcriptional activator from said fusion protein and thus allows cell survival and selection of positive cells.

31. The method of claim 30, wherein the host cell is a eucaryotic cell.

32. The method of claim 30 or 31, wherein the host cell is a yeast cell.

33. The method of one of claims 30-32, wherein said fusion protein comprises a known membrane protein and a transcriptional activator.

34. The method of one of claims 30-33, wherein the transcriptional activator is LexA-VP16.

35. The method of one of the claims 30-32, wherein said fusion protein comprises a known membrane protein and a secreted protein.

36. The method of claim 35, wherein said secreted protein is a protein with invertase activity or functional fragments of a protein with invertase activity.

37. The method of claim 36 wherein said secreted protein is a yeast invertase or functional fragments of a yeast invertase.

38. A secretase obtainable by a method of one of claims 30-37.

39. The secretase of claim 38 as diagnostic, preventive or therapeutic agent.

40. The method of claim 1 wherein said interacting protein to be identified is able to process a specific secretase and wherein

suitable host cells are transformed to express under suitable conditions a target protein of said secretase as a fusion protein with a secreted protein

said host cells transformed to express said fusion protein are further transformed to express an unprocessed specific secretase

said host cells transformed to express said fusion protein and said unprocessed secretase are further transformed with a library encoding putative processing proteins of said secretase

said host cells transformed to express said fusion protein, said unprocessed secretase and said library are cultivated under conditions allowing expression of said fusion protein, said unprocessed secretase and said library

said conditions furthermore allowing cell survival only in the presence of protein encoded by said library that processes said specific secretase to an

active secretase and thus allows release of said secreted protein from said fusion protein and thus cell survival.

41. The method of claim 40 wherein the host cell is an eucaryotic cell.

42. The method of claim 40 or 41 wherein the host cell is a yeast cell.

43. The method of one of claims 40-42 wherein the secreted protein is a protein with invertase activity or functional fragments of a protein with invertase activity.

44. The method of one of claims 40-43 wherein the secreted protein is a yeast invertase or functional fragments of a yeast invertase.

45. The method of one of claims 40-44 wherein the membrane protein is a transmembrane protein.

46. The method of one of claims 40-45 wherein the membrane protein is a type I transmembrane protein.

47. The method of one of claims 40-45 wherein the membrane protein is a type II transmembrane protein.

48. The method of one of claims 40-47 wherein said fusionprotein comprises a target membrane protein, a secreted protein and a transcriptional activator.

49. A protein obtainable by a method of one of claims 40-49.

50. The protein of claim 49 as a diagnostic, preventive or therapeutic agent.

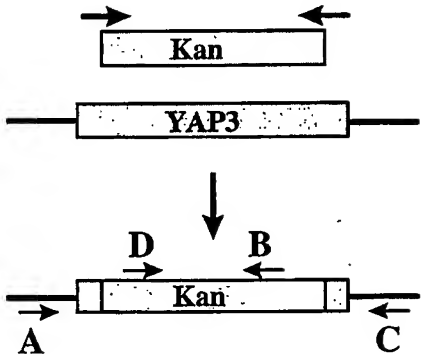
Fig. 1 Disruption of YAP3 and MKC7

Yap3p and Mkc7p:

- **two related GPI-linked yeast aspartyl proteases**
- **responsible for α-secretase-type cleavage of APP in yeast**

Zhang et al., BBA 1359 (1997) 110-22, Komano et al., JBC 273 (1998) 31648-51

Fig. 1A



Selection on kanamycin plates

Fig. 1B

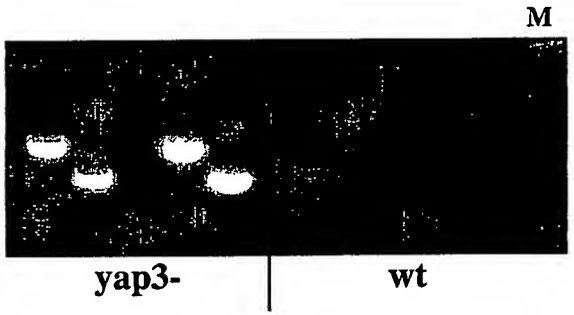


Fig. 1 (continued)

Fig. 1C

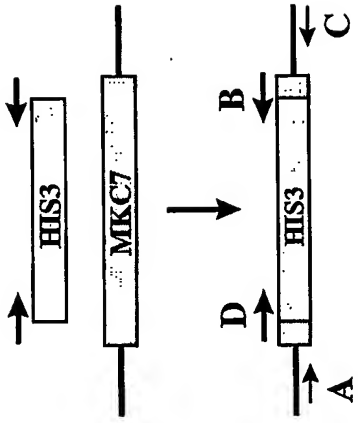


Fig. 1D

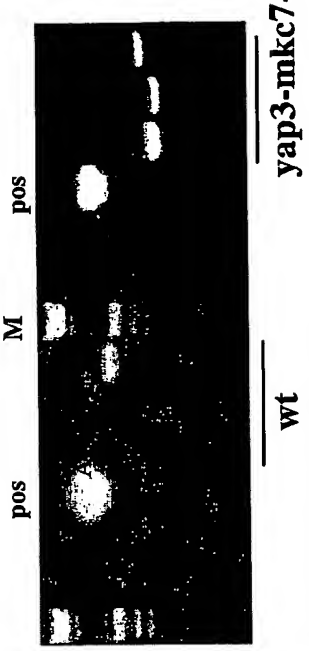
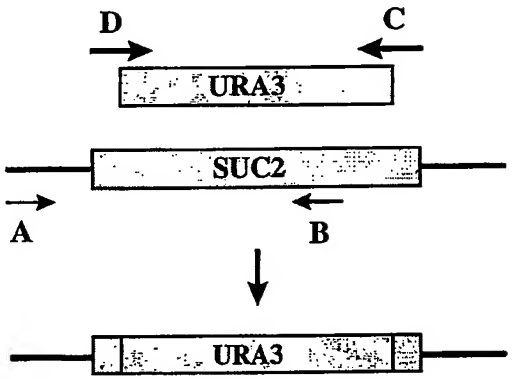


Fig. 2 Disruption of SUC2

SUC2 knock out in YAP3+ MKC7+ (α +) and yap3- mkc7- (α –) genetic background in *S. cerevisiae* strain JPY9

Fig. 2A



Selection on -Ura plates

Fig. 2B **Genomic PCR controls**

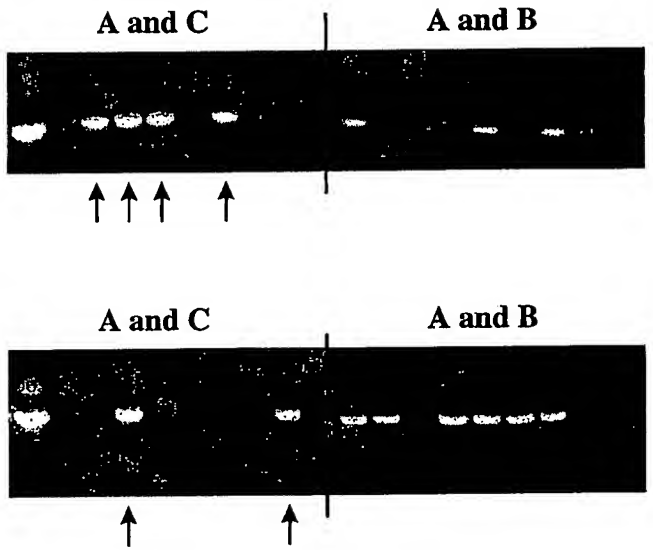
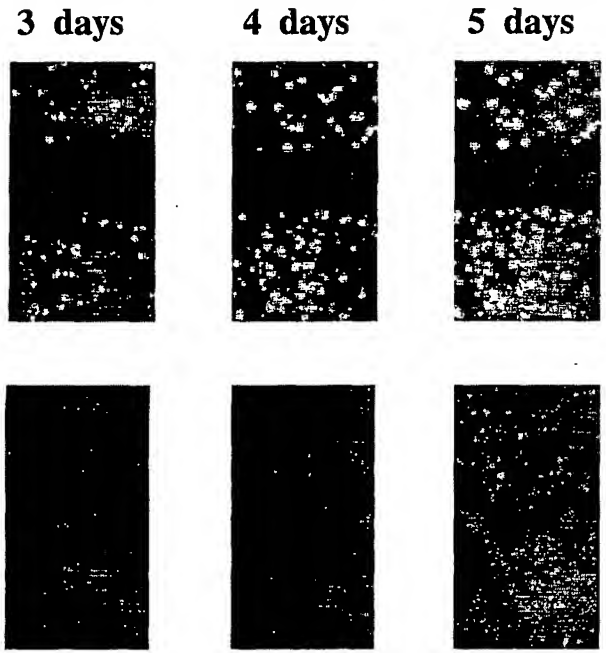


Fig. 3 Genetic test for suc2 disruption

Transformation of a SUC2 expression vector in JPY9 α +/SUC2- and JPY9 α-/SUC2- background



- **Growth independent of the MKC7/YAP3 genetic background**
- **Complementation of SUC2 in a suc2 - deficient strain results in normal growth**

Transformation 24.10.99

Fig. 4 SUC2-APP fusion with N-terminal truncated APP

Fig. 4A

α - background

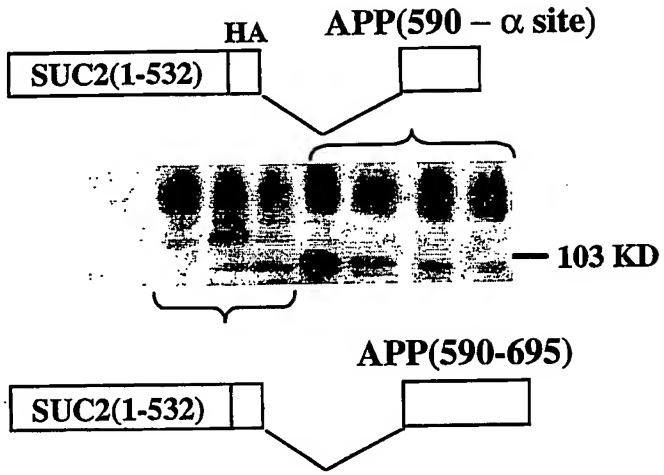
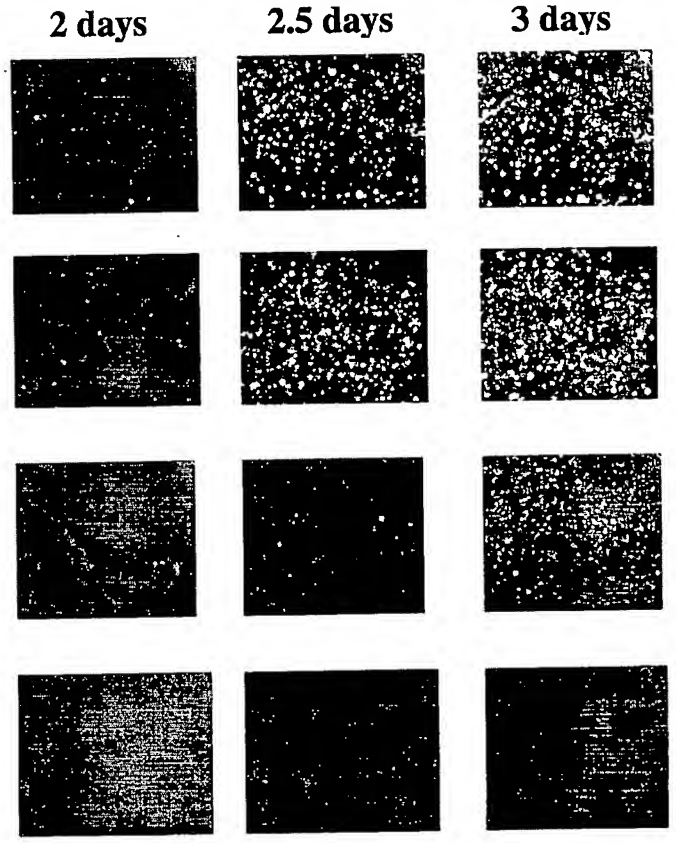
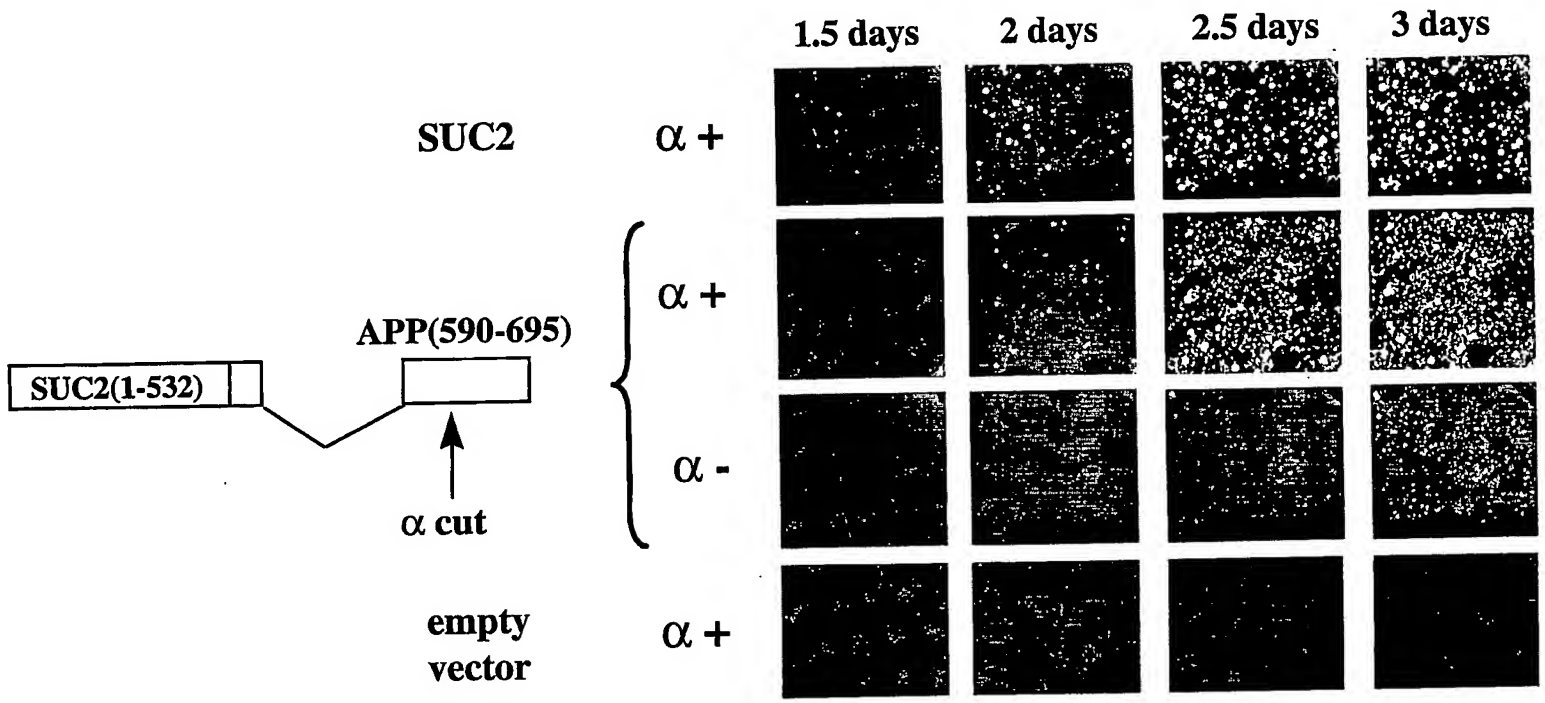


Fig. 4B



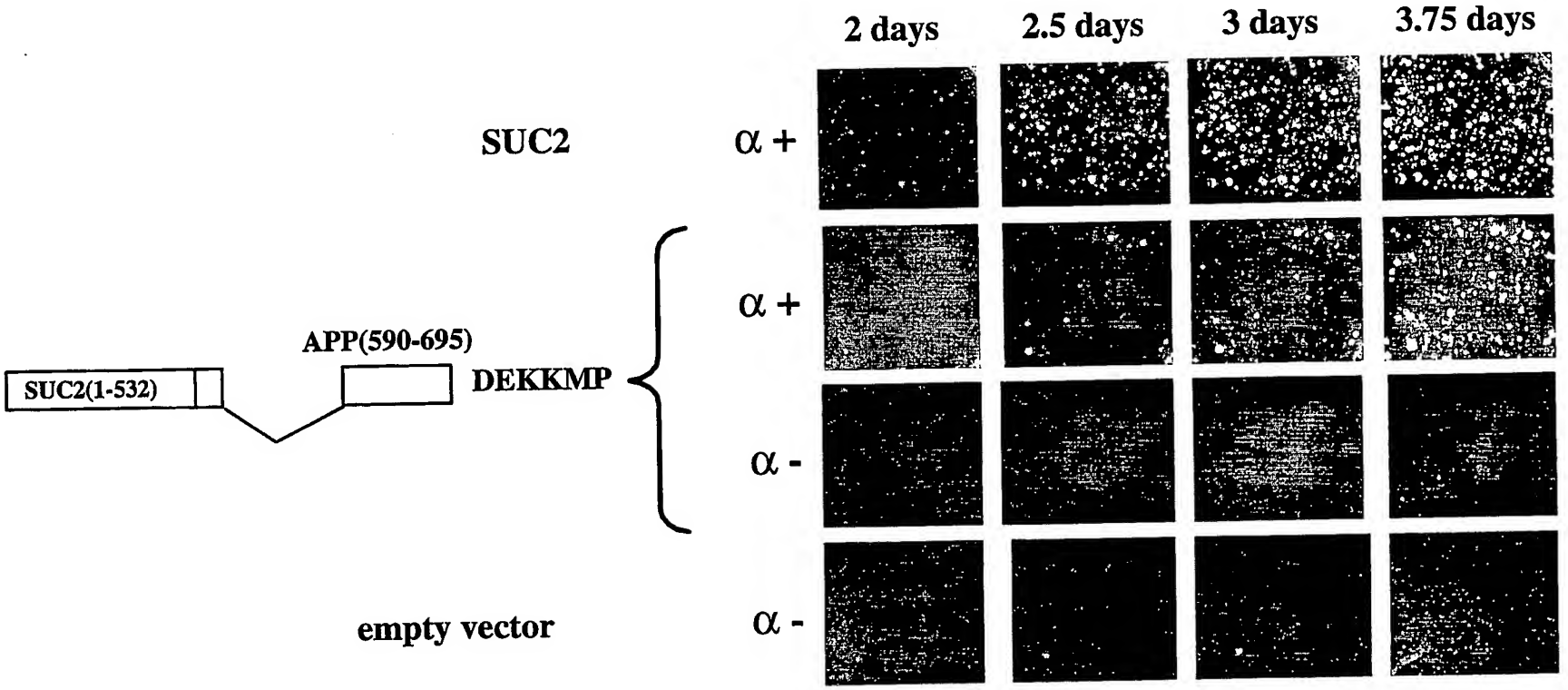
Transformation 5.11.99

Fig. 5 SUC2-APP(590-695) is activated in α + background



Transformation 10.11.99

Fig. 6 ER retrieval signal in α + and α– background



Transformation 10.11.99

INTERNATIONAL SEARCH REPORT

International Application No
PCT/IB 00/00552

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C12N9/64 C07K14/47 C12N9/26

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C12N C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

EPO-Internal, BIOSIS, WPI Data, PAJ, MEDLINE, CHEM ABS Data, SCISEARCH, BIOTECHNOLOGY ABS, EMBASE

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	WO 98 13488 A (SCHERING AG ;DYRKS THOMAS (DE); HAERTEL MARION (DE); TURNER JONATH) 2 April 1998 (1998-04-02) examples 1-4	1-10, 13-17, 20-27
Y	BOEHM JOHANNES ET AL: "Kex2-dependent invertase secretion as a tool to study the targeting of transmembrane proteins which are involved in ER fwdarw Golgi transport in yeast." EMBO (EUROPEAN MOLECULAR BIOLOGY ORGANIZATION) JOURNAL, vol. 13, no. 16, 1994, pages 3696-3710, XP000608640 ISSN: 0261-4189 page 3699, left-hand column, last paragraph -right-hand column, paragraph 2; figure 1	1-10, 13-17, 20-27

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[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

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"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
26 February 2001	23/03/2001

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	ALCONADA RODRIG.., A

Form PCT/ISA/210 (second sheet) (July 1992)

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
A	DIXON E P ET AL: "AN INVERSE MAMMALIAN TWO-HYBRID SYSTEM FOR BETA SECRETASE AND OTHERPROTEASES" ANALYTICAL BIOCHEMISTRY,US,ACADEMIC PRESS, SAN DIEGO, CA, vol. 249, no. 2, 1997, pages 239-241, XP000914815 ISSN: 0003-2697 the whole document	30-37
A	STEINER H ET AL: "AN IN VIVO ASSAY FOR THE IDENTIFICATION OF TARGET PROTEASES WHICH CLEAVE MEMBRANE-ASSOCIATED SUBSTRATES" FEBS LETTERS,ELSEVIER SCIENCE PUBLISHERS, AMSTERDAM,NL, vol. 463, 17 December 1999 (1999-12-17), pages 245-249, XP000946647 ISSN: 0014-5793 figure 1	30-37
A	DE 198 56 261 C (HOECHST MARION ROUSSEL DE GMBH) 30 March 2000 (2000-03-30) page 3, line 54 -page 5, line 24; examples 1-3	30-37
A	KAISER C A ET AL: "MANY RANDOM SEQUENCES FUNCTIONALLY REPLACE THE SECRETION SIGNAL SEQUENCE OF YEAST INVERTASE" SCIENCE,US,AMERICAN ASSOCIATION FOR THE ADVANCEMENT OF SCIENCE, vol. 235, no. 4786, 16 January 1987 (1987-01-16), pages 312-317, XP000610609 ISSN: 0036-8075 page 312, right-hand column, last paragraph; table 1	1-10, 13-17, 20-27, 30-37, 40-48
A	KOHORN BRUCE D: "Isolation of cDNAs encoding proteases of known specificity using a cleavable GA14 protein." METHODS (ORLANDO), vol. 5, no. 2, 1993, pages 156-160, XP000987043 ISSN: 1046-2023 figures 1,2	1-10, 13-17, 20-27, 30-37, 40-48

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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	KOMANO HIROTO ET AL: "Involvement of cell surface glycosyl-phosphatidylinositol-linked aspartyl proteases in alpha-secretase-type cleavage and ectodomain solubilization of human Alzheimer beta-amyloid precursor protein in yeast." JOURNAL OF BIOLOGICAL CHEMISTRY, vol. 273, no. 48, 27 November 1998 (1998-11-27), pages 31648-31651, XP002161371 ISSN: 0021-9258 the whole document	1-10, 13-17, 20-27, 30-37, 40-48

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

Continuation of Box I.2

Claims Nos.: 11, 12, 18, 19, 28, 29, 38, 39, 49, 50

Present claims 11, 12, 18, 19, 28, 19, 38, 19, 49 and 50 relate to secretases, secretase-interacting proteins, polypeptides interacting with a known transmembrane protein and a secretase-processing polypeptide defined by reference to a desirable characteristic or property, namely their ability to be detected in the assay as defined in claims 3-10, 13-17, 20-27, 30-37 and 40-49.
The claims cover all polypeptides having this characteristic or property, whereas the application provides support within the meaning of Article 6 PCT and/or disclosure within the meaning of Article 5 PCT for none of such proteins. In the present case, the claims so lack support, and the application so lacks disclosure, that a meaningful search over the whole of the claimed scope is impossible. Independent of the above reasoning, the claims also lack clarity (Article 6 PCT). An attempt is made to define the polypeptides by reference to a result to be achieved. Again, this lack of clarity in the present case is such as to render a meaningful search over the whole of the claimed scope impossible. Consequently, the search has not been carried out for those claims..

The applicant's attention is drawn to the fact that claims, or parts of claims, relating to inventions in respect of which no international search report has been established need not be the subject of an international preliminary examination (Rule 66.1(e) PCT). The applicant is advised that the EPO policy when acting as an International Preliminary Examining Authority is normally not to carry out a preliminary examination on matter which has not been searched. This is the case irrespective of whether or not the claims are amended following receipt of the search report or during any Chapter II procedure.

INTERNATIONAL SEARCH REPORT

Information on patent family members

ational Application No
PCT/IB 00/00552

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9813488 A	02-04-1998	DE 19641180 A	26-03-1998
		AU 4775797 A	17-04-1998
DE 19856261 C	30-03-2000	AU 1968000 A	26-06-2000
		WO 0034511 A	15-06-2000